PHYSIOLOGY.—*Anaerobiosis in* Australorbis glabratus: *Temperature effects and tissue hydration.* Theodor von Brand,[1, 2] National Microbiological Institute,[3] National Institutes of Health, Bethesda, Md.

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The relations between temperature and anaerobic survival of invertebrates have never been studied in detail. The few isolated data indicate, as expected, a lengthening of survival by lowering the temperature (for summary of these data see von Brand, 1946). Insofar as snails are concerned, only qualitative observations are available. Alsterberg (1930) found that *Lymnaea stagnalis* survived less than 2.5 days at 20°C, but longer than 7 days at 8–10 and at 0°C.

In the present paper quantitative studies of the influence of temperature on the anaerobic tolerance are presented, using the pulmonate snail *Australorbis glabratus*, the most important vector of schistosomiasis in the Western Hemisphere. Included are observations on changes in water content during anaerobiosis and during recovery therefrom. These latter studies were done because recent observations on chironomid larvae (Harnisch, 1954, a, b) had indicated that relatively large shifts in water content occur during anaerobiosis. It seemed therefore of interest to investigate the possible occurrence of a similar phenomenon in a representative of another phylum.

MATERIAL AND METHODS

A Venezuelan strain of *Australorbis glabratus* was used. The snails were laboratory reared and weighed between 200 and 400 mg each.

The snails were freed of excess water as described previously (Newton and von Brand, 1955) and weighed to the nearest mg. They were then placed in Warburg vessels of about 16 ml capacity containing 2 ml dechlorinated tap water. Anaerobiosis was established by flushing the manometers for 15 to 20 minutes with 99.99 percent Linde nitrogen further purified by passing over heated copper. At the end of the anaerobic period, the snails still alive were weighed and transferred to fresh dechlorinated tap water, the gaseous atmosphere now being air. When the preceding anaerobic temperature had been 35, 30, or 20°C, the snails were allowed to recover, usually for 7 hours, at the same temperature. Snails exposed to anaerobiosis at 10°C were kept postanaerobically at 20°C, because it is sometimes difficult at 10°C to recognize whether a snail is actually dead or only quiescent. At the higher temperatures dead snails could be recognized without difficulty: They were always more or less retracted into the shell and had freely hemorrhaged. At the end of the recovery period, the surviving snails were again weighed and then dried at 110°C until constant weight was reached. The water content was calculated by subtracting the dry weight from the initial, anaerobic, and postanaerobic fresh weights. It is evident that only the figures for the final water content are entirely correct. The initial water content and the water content after anaerobiosis are both slightly too high (the former more so than the latter), because the metabolized organic matter has been neglected. The error introduced is small, however, as indicated by previous metabolic experiments (von Brand, Baernstein, and Mehlman, 1950); it probably does not surpass 0.5 percent of the fresh weight.

RESULTS

Australorbis glabratus is a tropical snail and as such is not exposed to very low temperatures in nature. Previous respiration experiments (von Brand, Nolan, and Mann, 1948) have shown that it will tolerate short periods of exposure (2 hours) to 5.0° and 37.0C, while 0.3° and 41.0C were definitely harmful. In the present series of experiments

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[2] I am indebted to Mrs. M. O. Nolan for the contribution of all the snails used, to Dr. J. Buck for stimulating discussions of the topic, and to S. W. Greenhouse for the method of calculating and utilizing the slopes of the death curves to reflect the death rates.

[3] Laboratory of Tropical Diseases.

TABLE 1.—INFLUENCE OF TEMPERATURE ON ANAEROBIC TOLERANCE AND ON ANAEROBIC AND POSTANAEROBIC WATER CONTENT OF *Australorbis glabratus*

Temp.	Anaerobic period	Postanaerobic period	Snails	Percent dead after		Percent surviving	Initial water content in percent of fresh weight	Water content after			S*
				Anaerobic period	Postanaerobic period			Anaerobic period in percent of initial water content	S*	Postanaerobic period in percent of initial water content	
C	*Hours*	*Hours*	*Number*								
35	7.5	16	42	7	26	67	65.2 ± 2.2	117 ± 1.6	6.9	102 ± 1.6	0.9
35	16	7	49	61	29	10	64.0 ± 2.2	123 ± 3.7	1.7	113 ± 3.2	1.2
30	16	7	52	4	8	88	62.8 ± 0.5	115 ± 1.5	10.0	100 ± 1.7	0.0
30	23	7	55	29	13	58	60.8 ± 0.6	121 ± 2.2	9.6	103 ± 1.8	0.9
30	40	7	42	88	10	2	—	—	—	—	—
20	48	7	42	5	5	90	64.6 ± 0.2	116 ± 1.2	11.2	98 ± 1.1	1.8
20	64	7	42	19	12	69	63.6 ± 0.6	118 ± 1.8	5.5	102 ± 1.4	1.4
20	88	7	42	64	2	34	63.3 ± 1.2	118 ± 3.4	1.6	104 ± 2.6	0.6
10	72	7**	42	5	5	90	66.8 ± 0.2	103 ± 1.3	1.7	93 ± 1.1	5.4
10	120	7**	42	48	26	26	64.0 ± 1.0	106 ± 3.1	0.6	101 ± 2.8	0.1
10	144	7**	42	59	29	12	63.2 ± 1.6	108 ± 6.4	0.2	97 ± 4.8	0.1

* Significance: $\frac{M_1 - M_2}{\sqrt{E_1^2 + E_2^2}}$. If the resulting figure is greater than 2, the water increase is significant.

** Temperature of postanaerobic period: 20°C.

it was necessary to keep snails for much longer periods at various temperatures. To establish a base line for the anaerobic experiments, series of snails were exposed aerobically for 16 hours to 35°C and 144 hours to 10°C. None of the former died (35 specimens), while 3 out of 42 kept at 10°C succumbed. It is probable that within these temperature limits the anaerobic resistance could be tested without danger that "heat death" or "cold death" proper might obscure the results, although 10°C is probably close to the lower temperature limit tolerated. This point will be discussed below.

Table 1 shows that under all conditions investigated snails died during the actual anaerobic period and that a variable additional percentage was so damaged by lack of oxygen that death ensued during the subsequent aerobic "recovery" period. To assess the harmful effects of anoxia both death percentages were added and all further discussion is based on this total death figure.

Fig. 1 indicates that after an initial lag period the anaerobic deaths at each temperature followed a straight line. The length of the lag period increased with decreasing temperature. It is entirely possible that the straight line relationship does not hold for the last surviving snails; that is, it is possible that the curves may flatten out, thus leading to the frequently encountered sigmoid curves. This possibility, however, could not be tested experimentally without an impracticable wastage of experimental animals. As indicated by the "recovery deaths," the exact death point of all the snails under actual anaerobiosis cannot be determined with sufficient precision. However, an indication that a flattening out of the curves may occur can be seen in the location of the last two points of the 10° curve.

The data presented in Fig. 1 allow one to calculate the hours of anaerobiosis required to kill 50 percent of the snails. If the 50 percent death points are plotted, they scatter closely around a straight line (Fig. 2), a rather unexpected result. This relation

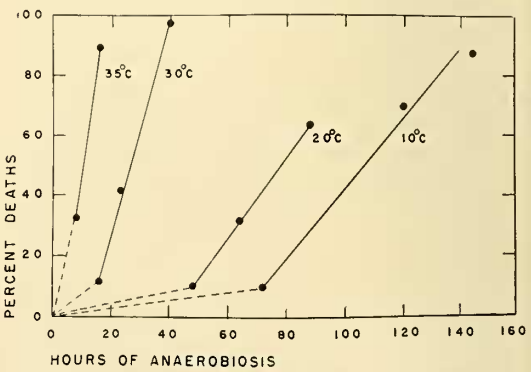

FIG. 1.—Death curves of *Australorbis glabratus* due to lack of oxygen at various temperatures.

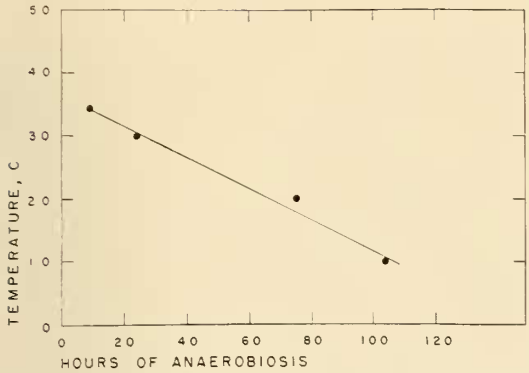

FIG. 2.—Influence of temperature on the time required to kill 50 percent of *Australorbis glabratus* specimens exposed to lack of oxygen.

means that a given decrease in temperature increases the time required to achieve a 50 percent kill by an equal length of time throughout the temperature range tested. Fig. 2 shows, for example, that each 5° decrease in temperature increases the time necessary to reach the 50 percent death point by about 20 hours. Linear relations between temperature and velocity occur in biological processes. Rather numerous literature quotations to this effect can be found in Belehradek's monograph (1935). The present case is no direct parallel, however. The time required to achieve 50 percent kill cannot be considered to give a rate in the strict sense (such as heart beat frequency would be) since it includes both the initial lag period during which no animal dies and also part of the linear death curves.

The slope of the linear portions of each death curve (Fig. 1) reflects the rate of death at the specified temperature. Death rates, expressed as percent death per hour, were computed from these lines by a graphical procedure and yielded the figures summarized in Table 2. Upon plotting these figures according to Arrhenius' equation two lines

TABLE 2.—ANAEROBIC DEATH RATE OF AUSTRALORBIS GLABRATUS IN PERCENT DEATHS PER HOUR

Temperature	Death rate
C	
10	1.21
20	1.35
30	3.58
35	6.71

result (Fig. 3), but the slopes of these lines can be considered as only approximate because of the few points available. It is, nevertheless, obvious that the temperature characteristics of asphyxiation death are quite different at low and higher temperatures. The temperature relationships of the aerobic respiration of *Australorbis*, on the contrary, gives only one straight line over an even greater range of temperatures (von Brand, Nolan, and Mann, 1948), with a μ value of approximately 17,400. Intersecting lines upon application of Arrhenius' equation are of course quite common (Crozier, 1924), but in most cases, the μ values are higher in the lower temperature range.

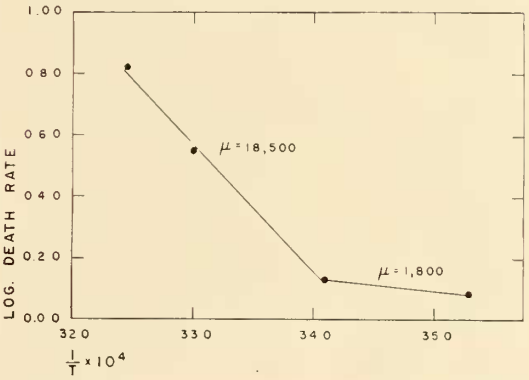

FIG. 3.—Anaerobic death rates of *Australorbis glabratus* expressed according to Arrhenius' equation.

The water content of anaerobically maintained snails (Table 1) increased rather markedly when the snails were subjected to anoxia at 35, 30, or 20°C. In all series where more than about 10 snails survived, the difference between initial water content and that found at the end of the anaerobic period was statistically significant. In most cases the surviving snails returned during the recovery period quite closely to their initial water content; that is, the surplus water was largely eliminated. The figures given are of course only of significance insofar as the state of hydration of the tissues is concerned; they are not indicative of the total amount of water exchange. The latter presumably would be greater as indicated by the fact that a portion of the anaerobic end products is actually excreted during the anaerobic period and also during a subsequent recov-

ery period (von Brand, McMahon, and Nolan, 1955).

While the data of Table 1 definitely prove a significant anaerobic hydration of the tissues at the higher temperatures, the anaerobic water increase at 10°C was very small and not statistically significant in any case. Enough snails survived at 10°C in at least two series out of the three done that a significant water increase would undoubtedly have been detected. It appears then that temperature has a definite influence on the anaerobic water regulation of the snail.

DISCUSSION

The ultimate mechanism of death by asphyxiation is not known. Lack of oxygen induces in any aerobic organism a chain of events, various links of which could be in themselves harmful. In anaerobically kept specimens of *Australorbis* specifically the following facts have been established (aside, of course, from a cessation of the oxidative processes connected with the aerobic oxygen consumption): A somewhat higher rate of carbohydrate consumption than observed in aerobic controls (von Brand, Baernstein, and Mehlman, 1950), excretion of carbon dioxide, the rate depending to some extent on the available polysaccharide stores (Newton and von Brand, 1955), excretion and accumulation in the tissues of small amounts of lactic acid and larger amounts of acetic and propionic acids (Mehlman and von Brand, 1951; von Brand, McMahon, and Nolan, 1955) and hydration of the tissues at temperatures above 10°C (present study).

The present investigation suggests that asphyxiation death may not always be due to one mechanism alone. If the concept of the "master reaction" is correct, the relations between anaerobic death and temperature discussed in the preceding section indicate that one "master reaction" is operative at 10°C and another above 20°C. It should be kept in mind that *Australorbis* is a tropical snail and that apparently 10°C is close to the lower temperature limit tolerated. It seems possible that cold itself puts a considerable stress on the organism which aggravates the stress due to lack of oxygen. A change in physiological response to anaerobiosis is indicated by the observations reported above in hydration differences at various temperatures. It would then seem possible that two competing mechanisms are involved in bringing about the relatively rapid death at 10°C: On the one hand, the lowering of the temperature to this level will undoubtedly lower the anaerobic metabolic rate[4] and thus tend to prolong life endangered by whatever phase of anaerobic metabolism is involved in asphyxiation death. On the other hand, the presumed "cold stress" would tend to shorten life. What mechanism may be involved here is not known. Actually, we are confronted by the riddle why some cold-blooded animals are confined to tropical and others to arctic environments. While some metabolic adaptations have been described (Scholander, Flagg, Walters, and Irving, 1953), it does not seem likely that they explain temperature segregation fully. It seems more probable that other factors, perhaps of a physicochemical nature, are of greater importance.

Coming back to the immediate problem, we see that the concept of a relative aggravation instead of an alleviation of anaerobic stress by low temperature in a tropical animal finds some support in the admittedly sketchy information available concerning the anaerobic temperature relationships of a cold-water snail, *Lymnaea stagnalis*. At 30°C 100 percent survived 6 hours, but only 9 percent 16 hours (von Brand, Baernstein, and Mehlman, 1950). At 20°C, the snails were dead before 60 hours had passed, while at 8–10, and at 0°C, they were still alive after 168 hours (Alsterberg, 1930). In other words, at 30 and 20°C the cold water snail was less resistant to lack of oxygen than the warm water snail, while the reverse held true at lower temperatures.

[4] The point was not checked experimentally because of technical difficulties. The most easily determined anaerobic process, carbon dioxide production, cannot be determined accurately in snails, especially not at various temperatures, because of the presence of the calcareous shell. The lactic acid production, another excellent yardstick in many cases, is not a practical approach in *Australorbis* because lactic acid is only a minor anaerobic end product. The polysaccharide consumption also does not lend itself to accurate determinations (unless very long series are done) because of marked fluctuations in the initial level.

SUMMARY

1. Anaerobic death curves were established at 35, 30, 20, and 10°C. At all temperatures there is an initial lag period, after which the death rate follows a straight line.

2. Upon application of Arrhenius' equation two lines result. The temperature influence is characterized by a very low μ value at 10°C, the lowest temperature tested, and a much higher one in the higher temperature range (20 to 35°C).

3. Between 35 and 20°C anaerobiosis induces a rather marked hydration of the tissues, while at 10°C only an insignificant increase occurs. The surplus water is rapidly excreted during a postanaerobic recovery period.

4. The idea is expressed that the relatively rapid anaerobic death at 10°C is due to an additive effect of anaerobic and cold stress.

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PROCEEDINGS OF THE ACADEMY AND AFFILIATED SOCIETIES

PHILOSOPHICAL SOCIETY

1365TH MEETING, OCTOBER 10, 1952

C. R. SINGLETERRY, of the Naval Research Laboratory, spoke on *Particle size from fluorescence depolarization*. The general understanding of colloid science has been hampered by too-ready extrapolation phenomena. Well-recognized advances have been made recently in the study of organic high polymers and proteins, but understanding of the soap colloids has proved more difficult because of the weakness of the forces of association. Spurred by the necessity for investigation of the emulsifying action of soaps in connection with the synthetic rubber program of World War II, McBain, Debye, and Harkins each contributed important techniques to the study of soaps and the structure of the "micelles" they form. These are groups of molecules with hydrocarbon ends as far removed from the water molecules as possible while their polar ends are in intimate contact with the water.

Oil-soluble soaps like petroleum naphthenates and sulfonates, when added to engine oil in small amounts prevent rusting of parts and reduce wear. Fundamental research at the Naval Research Laboratory has included work on oleic acid derivatives of known structure. Water, even in extremely small quantities, has a very large effect on the viscosity and other properties of these materials.

Harkins and Corrin noted a change in color of a dye when micelles are formed. Further investigation at the Naval Research Laboratory showed that Rhodamine B becomes fluorescent when adsorbed on soap micelles. When polarized light is used, the fluorescent light is found to be only 20 to 30 per cent polarized and the size of the micelles may be determined from the depolarization of the fluorescence. This depolarization results from Brownian rotation of the dye-containing micelle during the interval ($2–4 \times 10^{-9}$ sec) between light absorption and fluorescence emission. Micelles having a molecular weight of about 20,000 have been studied. (*Secretary's abstract.*)